METHOD FOR PRODUCING A TEMPLATE OF SEQUENCES OF CHEMICAL OR BIOLOGICAL MOLECULES FOR A CHEMICAL OR BIOLOGICAL ANALYSIS DEVICE

TECHNICAL FIELD

The object of the present invention is a method for producing in parallel a template for sequences of chemical or biological molecules for a chemical or biological analysis device.

The chemical or biological molecules may be amino acids or nucleotides in particular, the sequences then being peptides or oligonucleotides.

It is in particular applied to the production of a template of oligonucleotide probes with different sequences for analysis or diagnosis or research in genetics (mutations, polymorphisms, transcriptome).

STATE OF THE PRIOR ART

Novel chemical or biological analysis systems or devices are used for sequencing and studying gene expression. They comprise a group of molecular probes (oligonucleotides) attached to the miniaturized surface of a substrate in order to produce a biochip or DNA chip.

During the analysis of a sample, the extracted target nucleic acids are labelled and deposited on the template of probes. With hybridization (pairing between complementary strands of the DNA double helix) between the probes and the labelled targets, the sequences of the DNA sample may be located and identified.

Many production methods have been described and developed to improve the miniaturization and the density capacity of analysis sites on the chip.

Different techniques for grafting and immobilizing the probes (by covalent or electrostatic bonding) on different substrates (silicon, glass, polymer, gold electrodes ...) have been the subject of industrial research and development.

The templates of probes are then produced

- either by immobilization of presynthesized oligonucleotides by grafting them on a functionalized substrate or on a conductive polymer,
- or by synthesis of the oligonucleotide probes on the substrate *in situ*.

The in situ synthesis methods used hitherto require two different methods for addressing sites, which are i.e. manual addressing by a microrobot as described in US-A-5 474 796 [1] and photochemical addressing as described in WO-A-97/39 151 [2].

In document US-A-5 474 151 [1], functionalized sites are first formed on the substrate by using photoresists or masks for defining the different sites of the substrate, and the sequences of oligonucleotides are then formed on the functionalized sites by injecting reagents on the desired sites by means of a piezoelectric pump.

In document WO-A-97/39151 [2], the substrate first undergoes a treatment for providing it with functional groups protected with a photolabile protective group, and certain functional groups of the substrate are then

deprotected by selective irradiation through a mask to define the sites and then to have a nucleotide react on these sites to build the desired sequence.

In both cases, the in situ synthesis involves conventional coupling reactions through phosphoramidites, phosphites or phosphonates, for successive condensation of the suitably protected nucleotides. The synthesis cycle comprises the deprotection, coupling, blocking and oxidation steps and allows the oligonucleotide to grow from the surface of each site.

In photochemical addressing, the steps for deprotecting the nucleotides are carried out by means of light. Addressing of the sites is done by a lithography step (exposure to light through a mask). The photolabile protective group is removed by means of light, thus the coupling may be carried out later by dipping the substrate in a solution of an activated nucleotide (for example, with a phosphoramidite) in the presence of a catalyst, for example tetrazole.

This light-controlled combinatorial synthesis method requires production of a number of masks equal to the number of nucleotides which make up the oligonucleotide and is an expensive and tedious operation. If this method has the advantage of leading to very high density chips (resolution of the photolithographic method), the yield of the photochemical reactions (removal of photolabile groups) on the other hand, is never 100%, and the purity of the

oligonucleotide sequence made on the chip cannot be ascertained.

In the case of document US-A-5 474 796 [1], which uses the technique of jet printing (piezoelectric heads), for distributing the 4 activated basic nucleotides of DNA as well as the coupling reagents on different sites of the chip, the solid-phase synthesis on sites of about a hundred μm^2, with amounts of reagents of the order of a nanoliter, requires controlled atmospheric conditions (sensitivity to moisture and to oxygen) that are difficult to produce in the environment of a microdispenser and its associated equipment. Moreover, it requires solvents with high boiling points and reagents that are not very volatile.

In situ methods for oligonucleotide synthesis are even known that use masks to define the areas of the substrate that are to be subjected to successive synthesis cycles.

Thus, document EP-A-0 728 520 [3] describes a method for producing templates of oligonucleotide probes which uses a substrate including protected functional groups on its entire surface, designed for retaining the nucleotides, and a barrier material for defining the areas of the substrate to be modified. This material may be selected from lacquers, liquid oils, polyesters, polyurethanes and epoxy resins which are generally liquid prepolymers. It is deposited by different printing techniques. After depositing the barrier, deprotection in the vapour phase is performed

on functional groups not covered with barrier material. The use of a vapour phase is necessary because the barrier material used is soluble or entrained in the deprotection solvents. But the protection in the vapour phase poses problems with risk of toxicity in the environment and of attack by diffusion of materials.

Document US-A-5 658 734 [4] uses an entirely functionalized substrate and lithography techniques with the use of a photoresist for defining the areas of the substrate to be modified by inserting between the substrate and the photoresist a polymer which acts as a buffer. This method thus uses a photoresist which has to be deposited with a spin coater, on the layer of polymer deposited on the substrate, with each cycle for adding a nucleotide, which requires a lithography step in each cycle (two deposits with the spin coater, thermal annealing, exposure to light through a mask, development of the photosensitive resin, and then development of the buffer polymer). After the steps for deprotecting the functional groups of the substrate and adding a nucleotide through apertures made in the polymer, both layers are removed in a solvent. In this method, the polymer allows the photoresist to be isolated from the nucleotide biological layer. This system therefore requires, in addition to the lithographic and coating steps with the spin coater and, the production of a large number of masks.

Another drawback of this method lies in the risk of "lifting off" the protective layer during development of the protective polymer. In fact, by

diffusion of the solvent under the photoresist layer, detachment of the polymer may occur on the protected sites.

Specifically, the object of the present invention is a method for producing a template of sequences of chemical or biological molecules, for example of an oligonucleotide, with which the drawbacks of the methods described above may be overcome.

DISCLOSURE OF THE INVENTION

The method of the invention uses a selective localized deposit of a protective polymer on a structured substrate with functionalized microcuvettes. For a short while the protective polymer is capable of protecting the selected microcuvettes during a chemical reaction leading to coupling with the molecule of the next chain. Mechanical addressing by microdeposition on the selected microcuvettes, replaces photochemical or electrical addressing and lithographic or printing techniques used in the other methods of combinatorial synthesis, for example for producing oligonucleotide probes.

According to the invention, the method for producing a template of sequences of chemical or biological molecules formed of different chains of chemical molecules or M1, M2,.....Mn by in situ synthesis on a structured substrate with microcuvettes, comprises the following steps:

1) functionalization of the microcuvettes of the substrate by functional groups capable of forming a covalent bond with the molecules M1, M2,.....Mn;

2) deposit of a protection polymer on at least one of the microcuvettes by microdeposition of drops from said polymer to form caps of solid polymer in the selected microcuvette(s);

3) carrying out one or more chemical reactions to provide coupling of a first molecule M1 on the functional groups of the microcuvettes not covered with protection polymer;

4) removal of the protection polymer on the microcuvettes covered with this polymer, after the first reaction or one of the following reactions carried out in step 3); and

5) again carrying out steps 2), 3) and 4) to obtain the desired chaining sequences on each of the functionalized microcuvettes.

With the method of the invention, by suitable selection of the protection polymer and of a solvent for dissolving the polymer compatible with the procedures of an automated synthetizer, the synthesis of the oligonucleotide templates may be carried out in microcuvettes or microreactors etched in a substrate. To do this, an automatic synthesizer of oligonucleotides is combined with a mechanical addressing device (microdeposition robot or ink jet printing) of a protection polymer.

The mechanical addressing consists of selectively carrying out a deposit of polymer on the selected

microcuvettes forming a solid and sealed cover fitted onto the microcuvettes in order to determine and select active areas with respect to reagents used for adding monomer units from the next chain.

The method of the invention gives the possibility of selecting protective polymers protection, for which insolubility, seal and coating properties (controlled shape and thickness) are compatible and suitable for one or more steps of the synthesis cycle.

The method of the invention allows removal of the protection polymer, either after the first chemical reaction of the coupling cycle or after a complete coupling cycle.

The method of the invention allows the removal of this polymer cap to be included in the conventional automatic synthesis cycle of oligonucleotides, either in one of the rinsing steps or in an additional rinsing step in a solvent compatible with those conventionally used in the automatic synthesizer of oligonucleotides (acetonitrile, dichloromethane, tetrahydrofuran).

In the method of the invention, the microcuvette functionalization step 1) may be carried out by applying the following steps:

a) functionalizing the entire surface of the substrate and microcuvettes with functional groups;

b) depositing a protection polymer on all the microcuvettes by microdeposition of drops from said polymer to form caps of solid polymer on all the microcuvettes;

c) reacting the functional groups present on the substrate around the microcuvettes with a non-labile blocking group under the conditions used for the chemical coupling reactions and for the removal of the protection polymer; and

d) removing the protection polymer from all the microcuvettes.

Preferably, after step a) the functional groups are protected by a labile protective group, and deprotection of the functional groups is carried out after step b) and before carrying out step c).

Likewise, protection of the functional groups of the microcuvettes is preferably carried out upon implementing the method of the invention. In this case, the functional groups of the microcuvettes are protected by a labile protective group and step 3) comprises a first deprotection reaction of the functional groups.

According to the invention, the molecules M1, M2,.....Mn may be of different types. By way of example, they may be synthetic or natural, L- or D-amino acids, nucleotides (RNA or DNA), pentose or hexose. In the case of amino acids, the number of molecules M1, M2, ...Mn may be high. In the case of nucleotides, the number of molecules is more limited since it includes A, T (or U), G and C.

In this case, the nucleotides may be in the form of phosphoramidites, phosphotriesters or H-phosphonates according to the type of nucleotide synthesis used. These molecules include two reactive functions

(hydroxyl and a function with phosphorus) one of which, the hydroxyl function, is protected by a protective group, preferably identical with the protective group of the functionalized sites.

An appropriate protective group may be in particular a trityl or trityl-derived group, for example the dimethoxytrityl group.

When the method of the invention is used for producing oligonucleotide sequences by phosphoramidite synthesis, step 3) corresponds to a synthesis cycle and it consists of the removal of trityl protective groups, the coupling with a nucleotide, the reaction of the functional groups which have not been coupled with the nucleotide, with a blocking group and the oxidation into phosphate of the phosphoramidite group of the nucleotide.

In the method of the invention the deprotection (or detritylation), coupling, blocking, oxidation and protection polymer removal steps are carried out in an automatic synthesizer of oligonucleotides after having introduced a modification of the reaction chamber for treating substances of variable size.

The blocking group used in the blocking step is a group that is not labile under the conditions for removing the protective groups from the functional groups of the microcuvettes.

By way of example, for this blocking reaction a solution of dimethylaminopyridine (DMAP) in tetrahydrofuran and lutidine may be used.

In the method of the invention, the deprotection, coupling, protection material removal and possibly oxidation steps are carried out by dipping the substrate, either in the reaction chamber of the synthesizer or in the appropriate reagent baths. Only the step for addressing the microcuvettes which corresponds to a selective protection of the selected microcuvettes by the protection polymer, is carried out by microdeposition techniques.

Advantageously, the protection polymer is deposited on the desired microcuvettes in the liquid state by mechanical addressing by using selective microdeposition techniques of the piezoelectric actuator type, "pin and ring", ink jet printing, Archimedean screw and micropipetting.

This polymer may be in solution in a solvent and, in this case, the formed polymer film is annealed by heating the substrate to a temperature of 50 to 100°C, before carrying out step 3) or step c).

By addressing the polymer by microdeposition, microcuvettes may selectively be protect during one or more of the four synthesis reactions corresponding to the coupling of a nucleotide unit. Calibrated droplets of a polymer in solution may thereby be locally distributed on the selected microcuvettes. After evaporation of the solvent, they form sealed caps fitted onto the microcuvettes. This addressing is preferably done before the detritylation step or before the coupling step in the oligonucleotide synthesis cycle.

The originality of the method lies in the great possibilities of adapting the selection of the polymer and its protective role to the different steps in the oligonucleotide synthesis.

In the method of the invention, the selection of the protective polymer is very important and must meet a certain number of criteria.

First, this polymer must be able to be deposited by the microdeposition techniques mentioned earlier, by a solution of the polymer in a solvent, so as to form calibrated droplets which fit the microcuvettes. Annealing at a temperature of 50 to 80°C to remove the solvent and improve the adherence to the substrate is generally necessary.

In the ideal case where the polymer is protective during the four steps of the synthesis cycle, it must be inert and preferably free from reactive functions capable of being coupled to the synthesis molecules and reagents. It must not be soluble in the solvents used in the steps of the cycle and must exhibit impermeability with respect to these solvents.

In reality, it is sufficient for the polymer to act essentially as a protective cap in the detritylation step or in the coupling step. It is then possible to adapt and optimize the polymer-solvent pair for the reserved step.

It is preferable for the protective polymer not to include functions with free hydrogens of the OH, NH_2 or COOH type which, during the coupling step, would be capable of being coupled with molecules M1, M2,

.....Mn for example the nucleotides, to avoid consuming reagents unnecessarily which would then be removed with the protection polymer.

The polymer should be able to be removed by a solvent that is inert with respect to nucleotides and furthermore, it should exhibit physico-chemical properties in solution, such as viscosity and surface tension enabling it to be deposited on the microcuvettes by the selected microdeposition technique.

In the method of the invention, selection of the protection polymer is made according to the step(s) during which it should protect the microcuvettes, while taking into account its solubility in the solvents likely to be used for the oligonucleotide synthesis.

If it is desired to provide protection by the polymer only during the step for removing the protective groups from the functional groups, or during the detritylation step, it is sufficient to select a polymer insoluble in the detritylation reagents which generally comprise dichloroacetic acid (DCA) or trichloroacetic acid (TCA) or a Lewis acid of the $ZnBr_2$ type, at approximately 2-5% in a solvent such as dichloromethane, acetonitrile or toluene.

By way of examples of such polymers, the following may be mentioned: polyvinyl alcohols insoluble in dichloromethane, acetonitrile and toluene; polyhydroxystyrenes insoluble in dichloromethane; polystyrenes, polyvinyl carbazoles and polyimides

insoluble in acetonitrile; and polyethylene oxides insoluble in toluene.

If it is desired to provide protection of the microcuvettes by the polymer only during the coupling step, a polymer insoluble in the solvent used should be selected for coupling, generally acetonitrile.

For this purpose, a polymer may be used that is selected from the group formed of polymers and their derivatives of the polyvinyl alcohol, polystyrene, polyvinyl carbazole and polyimide types which are insoluble in this solvent.

The protection polymer is also selected according to the solvents capable of providing its removal by dissolution. Preferably, polymers are selected that are soluble in solvents compatible with those conventionally used in the automatic synthesizers of oligonucleotides, which are generally dichloromethane (for detritylation), acetonitrile (for the coupling proper) and tetrahydrofuran (for blocking and oxidation). However, other solvents such as acetonitrile and toluene may be used instead of dichloromethane for detritylation, without any loss in yield.

Polymers that may be used, their solubility properties in various solvents, their possible use in one or more steps of the method of the invention and their method of removal are given in the following Table 1.

Table 1

POLYMER	SOLUBILITY (solvents)	INSOLUBILITY (solvents)	POSSIBLE STEPS FOR PROTECTION	REMOVAL
PVA Polyvinyl alcohol	Water, DMSO	CH_3CN; THF; CH_2Cl_2; Toluene	Detritylation and coupling in CH_3CN, CH_2Cl_2, or toluene Blocking and oxidation in THF	Removal in water or DMSO
Polystyrene (PS)	Toluene Acetone CH_2Cl_2	CH_3CN	-detritylation in CH_3CN -coupling in CH_3CN	Removal in CH_2Cl_2
Polyhydroxystyrene (PHS) or Shipley XP 8843 resin	CH_3CN, THF, Alcohol, Acetone, PGMA	CH_2Cl_2	Detritylation in CH_2Cl_2	Removal in CH_3CN
Polyethylene oxide (PEO)	CH_3CN CH_2Cl_2 Water	THF, toluene	Detritylation in toluene	Removal in water CH_3CN CH_2Cl_2
Polyvinyl carbazole (PVK)	CH_2Cl_2 Chlorobenzene	CH_3CN	Detritylation and coupling in CH_3CN	Removal in CH_2Cl_2
Polyimide XU 5218 (Ciba Geigy)	Anisole and CH_2Cl_2	CH_3CN	Detritylation in CH_3CN and coupling in CH_3CN	Removal in CH_2Cl_2

Among the polymers in Table 1, polyvinyl alcohol is of interest as it may provide protection of the microcuvettes during all the steps of oligonucleotide synthesis. Furthermore, it may easily be used in microdeposition robots. On the other hand, its removal is not as easy because water and DMSO are not part of the synthesis solvents and will therefore require an additional step as well as drying.

Thus, in the case of the production of probes by using an automatic synthesizer (adapted sample support) coupled to a microdispenser robot (by capillarity, piezoelectric or of the "pin and spin" type), it is preferable not to use a protective polymer that is

soluble in water, which at the same time would require deposition and a removal step in an aqueous medium; this may be bothersome for continuation of the synthesis that is carried out in an anhydrous medium.

To avoid any additional drying treatment, for selective protection of the microcuvettes, selecting a polymer which will be removed in an anhydrous solvent and compatible with the cycle of solvents used in the automatic synthesizer is more appropriate.

For example, polystyrene may act as a selective protection polymer in the detritylation step carried out in acetonitrile (2% TCA) in which it is insoluble; the following coupling step is performed in acetonitrile (tetrazole); the polymer may then be removed by a rinsing cycle in dichloromethane in which it is very soluble. Furthermore, polystyrene is inert and does not have any OH functions capable of fixing active nucleotides and it also has a protective role in the coupling step (in acetonitrile).

Polyhydroxystyrene itself is also of interest as it is easier to deposit than polystyrene and easily removed in a conventional solvent of the synthesizer. On the other hand, it may only provide protection during the detritylation step in dichloromethane but may be removed by a rinsing cycle in acetonitrile before the coupling step in this solvent.

Poly(ethylene oxide) is also only appropriate for detritylation with the proviso that this be conducted in toluene but it is easily removed in CH_3CN or CH_2Cl_2.

Polyvinyl carbazole and polyimide may provide protection during the detritylation and coupling steps in CH_3CN then be removed by means of CH_2Cl_2 which is part of the solvents used in the synthesizers. However, deposition of these polymers is more difficult.

The removal of the protection polymer may be done directly in the synthesizer, in a rinsing step introduced in the protocol of the automatic synthesizer cycle.

For example, the conventional solvent used in the detritylation step, dichloromethane, may be replaced by solvents such as acetonitrile, toluene, xylene, or even a halogenated aromatic solvent without any loss in effectiveness of the detritylation reaction by the acid reagent usually used (TCA or DCA).

The method of the invention has numerous advantages as compared with the methods of the prior art.

In fact, as compared with the method of reference [2] which uses photochemical addressing for in situ synthesis or that of reference [4] which uses a lithographic method, the method of the invention avoids the necessity of producing masks, i.e. lengthy and expensive operations, because it is a matter of producing a number of masks equal to the number of molecules M1, M2,.....Mn that form the sequences. Furthermore, in the case of photochemical addressing, deprotection is not complete and the longer the sequence, the lower the probability of obtaining the desired sequence. Consequently, redundant sequences are

formed on the substrate, this is not required in the invention where deprotection is complete.

As compared with the method of reference [1], the method of the invention is easier to implement as the coupling reactions may be carried out with an excess of reagents while, according to reference [1], the coupling reactions are carried out with several nL of reagents directly on the site to be modified under specific conditions, i.e. in an atmosphere of nitrogen or argon.

As compared with reference[3], the method of the invention is easier to implement as the microcuvettes, or synthesis sites, are defined beforehand, the protection is tighter by using caps of solid polymer and the deprotection reactions may be carried out in the liquid phase directly in an automatic synthesizer and not in the vapour phase.

In the method of the invention, deposition of the protection polymer on the selected microcuvettes by microdeposition does not require special precautions, the only proviso is that the droplet of polymer cover the microcuvette to be protected.

Other characteristics and advantages of the invention will become more apparent upon reading the description that follows, of course, given by way of illustration and non-limiting with reference to the appended drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A-1G illustrate the different steps of the method of the invention for producing a template of oligonucleotide probes.

Fig. 2 illustrates the outline of a microcuvette of (650 x 650 x 25 μm^3) filled with polymer.

Figs. 3 and 4 are top views of microcuvettes that may or may not be filled with polymer.

DETAILED DISCLOSURE OF THE EMBODIMENTS

To implement the method of the invention, one starts with a glass or preferably silicon substrate, structured with microcuvettes from 150 µm to several mm apart, but preferably several hundreds of µm and a depth of the order of 10 µm. These calibrated microcuvettes are produced by conventional photolithographic and etching methods.

Substrate 1 provided with microcuvettes 3 is seen in Fig. 1A. Microcuvettes 3 are functionalized by hydrophilic functional groups 5 such as hydroxyl groups, which are protected by protective groups 7. These protective groups may be dimethoxytrityl groups, conventionally used in oligonucleotide synthesis.

When the substrate is a silicon wafer, functionalization of the sites may be carried out in the following way.

The surface of the silicon plate is first oxidized to SiO_2 thermally, for example to a thickness of 5,000 Å, then activated by cleaning with oxidization in

alkaline or acid medium into Si-OH. Next, functionalization of the complete surface of the substrate is carried out by treatment with a functionalized silanization agent of the aminosilane or epoxysilane type with a linker molecule or spacer arm of the glycol type, for example a silane including a N(bis-hydroxymethyl) group and a spacer arm for example of the polyethylene glycol type. The introduction of such a spacer between the silane of the substrate and the oligonucleotide that will subsequently be synthesized, is of interest as the hybridization yield may be increased during use. The reactive functions are protected by a group such as dimethoxytrityl (DMT).

Protection of all the microcuvettes is then carried out by polymer caps deposited in the form of calibrated droplets with a robot for microdeposition by jets.

After drying the droplets, the ODMT functions on the surface of the substrate not protected by the polymer are deprotected by detritylation in an acid medium (TCA). These reactive functions of the external surface of the microcuvettes are blocked (capping) in order to limit the in situ synthesis of the oligonucleotides in the microcuvettes exclusively, and then the polymer is removed from the entire substrate by rinsing in a solvent.

Next, step 2) is carried out by depositing a protection polymer 9 on at least one of the functionalized microcuvettes of the wafer, with the aid

of a micropipette 11 such as a dispenser robot or by jet printing, as illustrated in Fig. 1A.

Fig. 1B illustrates the following step which consists of removing the protective groups 7 from the functional groups 5 on the microcuvettes 3 of the wafer not covered with protection polymer 9. Thus, a wafer is obtained in which the microcuvettes 3 not protected by the protection polymer 9 include deprotected hydroxyl functional groups 5. This step may be carried out by dipping the wafer in an acid solution, for example a solution of trichloroacetic acid in dichloromethane CH_2Cl_2, to remove the trityl groups.

Fig. 1C illustrates the following coupling step 3) of a molecule M1 by reaction with the hydroxyl functional groups 5 on the microcuvettes 3 not protected by the protection polymer 9. If it is desired to produce a template of oligonucleotide probes, the molecules M1 are formed by nucleotides such as phosphoramidites, phosphonates or phosphates according to the synthesis method for producing the probe. In this step, the coupling of the first nucleotide M1 (phosphoramidite) with the hydroxyl groups 5 is carried out by immersing the wafer in a solution of the activated nucleotide M1 in acetonitrile in the presence of tetrazole. Thus the structure illustated in Fig. 1C is obtained.

Fig. 1D illustrates the following step, according to which the remaining free hydroxyl groups that have not reacted with the molecule M1 are blocked, so that they cannot subsequently react with the other

nucleotides used for the different successive couplings. This reaction may be carried out by placing a blocking group 11 for example by dipping the wafer in a solution of dimethylaminopyridine (DMAP) in tetrahydrofuran (THF).

In the case of the production of an oligonucleotide sequence by the method using phosphoramidites, an oxidation is subsequently carried out on the trivalent phosphorus atom introduced during the coupling of the preceding step (Fig. 1C) into a more stable pentavalent phosphorus atom. This may be carried out by dipping the wafer in an oxidizing solution of iodine in a mixture of water, pyridine and tetrahydrofuran (THF).

After this oxidation step, the following step 4) is carried out with removal of the protection polymer 9 from the unmodified microcuvettes. Thus, the structure illustrated in Fig. 1E is obtained. This removal of the protective polymer 9 may be carried out by dissolution in an appropriate solvent by dipping the wafer in this solvent.

Thus, apart from the protection polymer deposition step, the steps of the method of the invention are carried out by dipping the wafer in the appropriate reagents and solvents, followed by rinsing in different solvents if necessary. These operations may be done in containers with a controlled atmosphere. These different steps by dipping in the appropriate reagent baths may advantageously be carried out in a reaction

cell, connected to an automatic synthesizer of oligonucleotides.

Furthermore, the reagents used are in great excess as compared with the molecules on the solid substrate, which provides the advantage of obtaining reactions with a conversion level of virtually 100%, which was not the case with reference method [1] where the reactions are carried out by micropipetting the reagents on the selected sites.

After this first step for modifying the selected microcuvettes with a molecule M1, other microcuvettes may be modified with molecule M2 .....or Mn. In this case, protection of the already modified microcuvettes is first carried out by the protection polymer 9. Thus, the structure illustrated in Fig. 1F is obtained. Of course, other microcuvettes of the substrate may also be covered with a protection polymer 9 even if they have not been modified in the preceding steps by molecule M1. After this operation, the protective groups 7 are then removed from the hydrophilic functional groups 5 of the microcuvettes not covered with protection polymer 9, another nucleotide M2 is then coupled on the functional groups 5 by operating in the same manner as earlier (Figs. 1B and 1C). Fig. 1G illustrates the obtained structure. After this operation, blocking of the hydroxyl groups that have not reacted may be carried out, and then oxidation of trivalent phosphorus into pentavalent phosphorus, and removal of the protective polymer 9 from the microcuvettes not modified by molecules M2.

Next, these different operations are carried out again on the selected microcuvettes to obtain the desired chaining of molecules M1, M2,.....Mn i.e. oligonucleotides with different chaining sequences on the different microcuvettes of substrate 1.

By way of example, a template of oligonucleotide probes was produced on a silicon substrate including microcuvettes of 100 x 100 x 30 μm etched in the silicon. Functionalization of the microcuvettes was carried out with a silanization agent formed by N,N-(bishydroxyethyl)aminopropyl trimethoxysilane wherein the OH groups are protected by a dimethoxy- or monomethoxytrityl group.

An exemplary embodiment of a template of two oligonucleotide sequences is described hereafter.

One starts with a structured substrate with microcuvettes using the conventional microelectronics methods, i.e., deposition, photolithography and etching. An outline of microcuvette 3 is shown in Fig. 2.

After cutting out a silicon substrate into a 2 x 2 cm^2 chip, the cleaning (1), silanization (2) and hexaethylene glycol arm branching (3) procedures are carried out as follows:

1) The substrate is incubated for 2 h at ambient temperature in a solution of NaOH (1 g NaOH, 3 mL deionized (DI) water, 4 mL 95% EtOH) and then rinsed with DI water and dried with a blower

2) The substrate is incubated overnight at 80°C in a solution including 1 mL of

3-glycidoxypropyltrimethoxysilane, 3.5 mL of toluene and 0.3 mL of triethylamine. It is rinsed with acetone, dried with the blower, and then left for 3 h at 110°C.

3) The substrate is incubated overnight at 80°C in a solution containing 15 mL of hexaethylene glycol and 9 µL sulphuric acid, and then it is rinsed in acetone and dried with the blower.

The substrate is then positioned in a specific part connected to an automatic synthesizer of oligonucleotides EXPEDITE 8909. The synthesis procedure makes use of phosphoramidite chemistry and consists of four steps: detritylation, coupling, capping, and oxidation. The procedure used is on the scale of 1 µmole.

The first seven parents are made in the 3' ATC TCA C 5'automatic synthesizer. The substrate is taken out of the synthesizer and is brought on a robot for depositing the polymer (Cam/alot) or the polymer is dispensed on half of the cuvettes. The outline of the filling with polymer 9 into microcuvette 3 is illustrated in Fig. 2. A top view of the microcuvettes with the light microscope is shown in Fig. 3 for sizes of 300 µm to 600 µm apart, and in Fig. 4 for sizes of 200 µm to 400 µm apart. Some microcuvettes are empty.

The substrate is baked at 85°C for 1 min 30 secs on a heating stage. The substrate is put back into the synthesizer to carry out the coupling of base C on the half of the cuvettes not covered with polymer. The polymer is removed in water at 85°C. A new deposition of

polymer is carried out on these latter cuvettes. After baking, the substrate is placed in the synthesizer to carry out coupling of base T in the other cuvettes. After this coupling, the polymer is removed and the substrate is placed in the synthesizer where all the cuvettes receive the following couplings: C AAA TAG. At the end of this step, half of the microcuvettes contain sequence No. 1: -3′ ATC TCA CTC AAA TAG 5′- and the other half sequence No. 2: -3′ATC TCA CCC AAA TAG 5′-.

These two sequences only differ by one base. The substrate is taken out of the synthesizer and is incubated in a solution of NH_4OH for 45 min at 60°C, then it is rinsed with DI water and dried with the blower in order to remove the protective groups from the nucleotide bases.

Hybridization is carried out with the complementary target of the No. 2 probe: -3′ CA TAG AGT GGG TTT ATC CA 5′- including a biotin group at 5′. The substrate is put into a solution containing 690 µL of H-7140 hybridization buffer from Sigma and 10 µL of the target at 1 OD (amount of oligonucleotides which, when dissolved in water, results in an absorbance of 1 measured at 260 nm in a tank with an optical path equal to 1 cm) and is incubated at 40°C for 1 h with slight stirring, and then 2 rinses are performed in a SCC 2X buffer bath for 1 min, and in an SCC 0.2X buffer bath for 1 min in order to remove the non-hybridized targets. The substrate is then dried with the blower. The coupling of the biotin with the streptavidin group

including the Cy3 fluorophor group is done in a solution containing 5 µL of Cy3 streptavidin buffer and 700 µL of PBS, TW, 0.5M NaCl buffer at ambient temperature in the dark for 10 to 15 min. The substrate is then rinsed in a PBS, TW 0.5M NaCl buffer bath for 1 min, and then in a PBS buffer bath and dried with the blower.

The fluorescence image obtained after hybridization on the fluorescence confocal microscope GS 3000 indicates that only hybridization with the complementary probe occurred.

Cited references

[1]: US-A-5 474 796

[2]: WO-A-97/39 151

[3]: EP-A-0 728 520

[4]: US-A-5 658 734